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PRINCIPAL INVESTIGATOR: William G. Cance, M.D.

CONTRACTING ORGANIZATION: University of North Carolina at
Chapel Hill
Chapel Hill, North Carolina 27599-4100

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Chapel Hill, North Carolina 27599-4100

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The central aims of this project are to characterize novel kinases in breast cancer biology. During this first year of the award, we have centered our work on the Rak tyrosine kinase. Rak is a nuclear tyrosine kinase identified from breast cancer cells, which is a potent growth suppressor gene. We have demonstrated Rak's growth inhibitory effects in several different breast cancer cell lines. We have also explored the mechanism of the growth inhibition to determine whether this is related to inhibition of the CDC2 kinase, and which specific sequences of Rak are required for the growth inhibition. Our current work is focusing on characterization of an inducible system of Rak expression in breast cancer cell lines to further analyze the mechanism behind this growth arrest. We also are developing monoclonal antibodies to Rak to study both the levels of Rak expression and its nuclear localization in large samples of human breast cancers.

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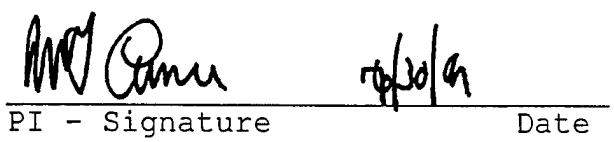

PI - Signature Date

TABLE OF CONTENTS

I. INTRODUCTION

This research grant focuses on the biology of protein kinases in human breast cancer. It appears that several protein tyrosine kinases are implicated in the progression of human breast cancer, including HER2/*neu* and EGFR. We have isolated novel protein kinases from human breast cancer cells using low stringency PCR amplification of the consensus sequences contained within their kinase domains. The current work has focused on the Rak tyrosine kinase. Rak is a novel nuclear tyrosine kinase identified in breast cancer tissues and cell lines that has structural homology to the Src tyrosine kinase, with SH2 and SH3 domains at its amino terminus. The Src family of tyrosine kinases localize primarily to the cell membrane, where they are thought to play a role in mitogenic signaling. The sequence of Rak revealed that it does not have an amino terminal region which targets its localization to the cell membrane. Rather, Rak has a putative nuclear localization sequence within its SH2 domain, and we have shown that Rak localizes to the nucleus. We suspected that the novel localization of Rak suggested that Rak might have a different role from other Src-related kinases, and we subsequently found that Rak has growth inhibitory activity in a number of cell lines. The continuation of that work will be described below.

In addition, we have found that localizes to chromosome 6q21, a region that is frequently deleted in breast and ovarian cancers. We plan to pursue the significance of this chromosomal localization later in the time frame of this project, and will discuss our plans below.

As described in the cover letter, I have taken over the management of this project following Dr. Edison Liu's departure to the National Cancer Institute. Because our original Annual Report was deemed unsatisfactory, I have rewritten the report, and will describe our present data and rationale for my current approach to the project. Some of the Specific Aims described in the original grant application will not be continued, because my lab does not have the expertise to perform the experiments, and the experiments were criticized by the study section. Specifically, Technical Objective 4 called for a screen for mutations to the Cyclin H gene in primary breast tumors. My lab has neither the reagents nor the experience to efficiently perform these experiments. In Technical Objective 5, transgenic mice were to be prepared in which the CDK7 and Cyclin H genes were to be expressed from the mammary-specific WAP promoter. Again, my lab is not prepared to perform these experiments efficiently.

However, I have extensive experience in protein kinases in human breast cancer, including analysis of gene expression in tumors and the targeting of genes that are expressed in breast tumors. I have been involved in the Rak project since its inception, and published the initial 2 reports on the gene and its characterization. In addition, my lab has published extensively about the expression and activity of the Focal Adhesion Kinase (FAK). These experiments are exactly the proposed experiments in Specific Aim 1, and I will describe our progress toward these objectives. My overall goal with this project is to direct it more towards the diagnosis of breast cancer, and the development of novel therapeutic strategies for breast cancer treatment.

II. BODY

A. Technical Objective 1: We will analyze the nature of Rak's growth inhibitory activity

In the early stages of these experiments, we sought to express the Rak tyrosine kinase in NIH 3T3 cells. We thought that the structural similarity of Rak to tyrosine kinases of the Src family was a hint that Rak might be a transforming gene. However, we found that Rak could not be stably expressed in NIH 3T3 cells, and inhibited their growth. We performed a colony formation assay to demonstrate this activity. It is worthy of note that the colonies that were transfected with Rak, but which emerged from selection, no longer expressed Rak.

TABLE I. Expression of Rak in NIH 3T3 cells

	Expt			
	1	2	3	ave
pcDNA3	131	173	112	100%
pc3-Rak	51	81	48	43%
pc3-Rak192	nd	78	53	46%

Table I. NIH 3T3 cells were transfected with a control plasmid pcDNA3, the same plasmid containing the Rak cDNA (pc3-Rak), or a plasmid containing a truncation mutant of Rak (pc3-Rak192), which does not contain the Rak kinase domain.

We subsequently found that Rak formed a physical association with the protein product of the Retinoblastoma tumor suppressor gene (pRb). This suggested that Rak might inhibit growth by somehow cooperating with pRb. Therefore, Rak should no longer inhibit growth in cells that lack pRb. This was Technical Objective #1b in the original proposal, and to test this, we obtained primary mouse fibroblasts from Dr. Tyler Jack's lab that were Rb(+/+) or Rb(-/-) and transfected these cells with a plasmid which directs the expression of Rak. While the growth of these cells was clearly disrupted, we were not able to quantitate this effect because the cells did not form colonies which could be reliably counted.

We then resorted to the use of the osteosarcoma cell lines U20os and Saos-2, which are Rb(+) and Rb(-), respectively. When cells were transfected with the Rak-expression plasmid, there was an inhibition of growth that was similar to our earlier findings. In Rak-transfected cells, colony formation was inhibited by approximately 60%. This growth inhibition was independent of the presence of the Rb gene product, as Rak inhibited growth equally in U2-os and Saos-2 cells.

TABLE II. Expression of Rak in Rb(+) and Rb(-) osteosarcoma cell lines

	1	2	3	ave
U2os (Rb+)				
vector	219	157	186	100%
Rak	93	79	85	43±8%
Saos-2 (Rb-)				
vector	278	182	162	100%
Rak	108	105	69	47±10%

Table II. Cell lines were transfected with the same plasmids as described in Table 1. Cells were maintained in medium containing 400 ug/ml of Geneticin to select for transfected cells. Emerging colonies were stained with crystal violet and counted. The results shown represent an average of three independent experiments.

Although we were not scheduled to begin work on Technical Objective 1c until this year, we have begun work on this project for several reasons. (1) We have found that Rak is expressed primarily in epithelial cells. We reasoned that epithelial cells may have a mechanism for regulating Rak that is absent in the mesenchymal cells from our earlier studies. For that reason, we felt that it was preferable to study Rak in a cell line which normally expresses Rak. (2) In the original cloning of Rak, we found that Rak is expressed in approximately 1/3 of primary breast tumors. We wished to know whether Rak had a biological activity in breast tumor cells, and whether this could help us understand why Rak was expressed in such a limited subset of tumors. Thus, we felt that it was of primary importance to determine whether Rak could be expressed in breast cancer cells.

As an initial set of experiments, we transiently transfected Rak into BT-20 cells. This was the cell line from which Rak was originally cloned. We used the Lipofectamine reagent from GibcoBRL to introduce the plasmid into the cells. Initially, the standard transfection procedure caused a large amount of toxicity, and during the course of the experiment, most of the cells died, whether they were transfected with Rak or a control plasmid. However, when we reduced the amount of Lipofectamine reagent from 40 µl to 10-20 µl per transfection, we no longer observed any toxicity, and cells transfected with a control plasmid were viable after 72 hours. Thus, the final procedure was to transfect 1X106 cells with 5µg plasmid and 20 µl Lipofectamine for 3 hours, then replace with fresh media and count the number of adherent cells after 72 hours.

Surprisingly, by 72 hours, approximately 50% of the cells transfected with the Rak expression plasmid had detached from the dish and appeared to have died (Table III). It is important to note that in our earlier experiments in mesenchymal cell lines, transfection with Rak did not have a short-term phenotype. Rather, the cells transfected with Rak appeared morphologically identical to cells transfected with a control plasmid. The growth inhibition phenotype was not evident until the cells had been maintained in culture and colonies had emerged from selection. For this reason, we were surprised to detect such an immediate phenotype in BT-20 cells.

	pBK-CMV		pBK-Rak	
Expt #1	$1X10^6$	(100%)	$5X10^5$	(50%)
Expt #2	$1.2X10^6$	(100%)	$7.2X10^5$	(60%)
Expt #3	$1.1X10^6$	(100%)	$5.4X10^5$	(49%)
Expt #4	$6.8X10^5$	(100%)	$3.8X10^5$	(56%)
	pcDNA3		pc3-Rak	
Expt #1	$5.6X10^5$	(100%)	$2.4X10^5$	(43%)

Table 3. Transient transfection of Rak into the BT-20 breast cancer cell line. Cells were transfected as described in the text, and the number of adherent cells were counted after 72 hours of growth in complete culture medium. Each experiment is the average of at least three separate transfections.

We performed several experiments to confirm that this was not simply a transfection artifact. First, we repeated the experiment four separate times and obtained similar results each time. For the first two experiments, we used an initial preparation of the Rak cDNA cloned into the pBK-CMV plasmid from Stratagene, with the pBK-CMV plasmid alone as a control. We considered that the inhibition that we observed following transfection with the pBK-Rak might be due to some impurity in our DNA preparation, and prepared new batches of both plasmids. These new preparations were used for Experiments 3 and 4. We also considered that this rapid loss of adhesion might be due to an oddity of the pBK-CMV plasmid, so we transfected cells with the Rak cDNA cloned into the pc-DNA3 plasmid from InVitrogen. Again, we observed a similar loss of viability following transfection with the Rak plasmid.

Next, we wished to determine whether the rapid loss of adherence that we observed could only be detected in the BT-20 breast cancer cell line. We transfected the MCF-10 and MCF-7 cell lines with the pBK-CMV and pBK-Rak plasmids. We also used a mutant of Rak containing a point mutation at position 262. This is a lysine-to-arginine mutation at the binding pocket for ATP. In other related kinases, this serves to inactivate kinase activity. This plasmid was called pBK-RakKD for kinase-dead, and the lack of kinase activity was confirmed by transfection of this plasmid into COS-7 cells (data not shown).

Following transfection into MCF7 and MCF10 cells, we again observed a loss of adherence and viability in cells transfected with Rak. Each of the experiments was performed at least three times. In MCF7 and BT-20 cells, the pBK-RakKD plasmid lead to essentially the same loss of adherent cells as the original Rak expression plasmid. However, in MCF-10 cells, there was less of an effect, and the pBK-RakKD plasmid did not cause a substantial loss of adherence. These results are shown in Figure 1.

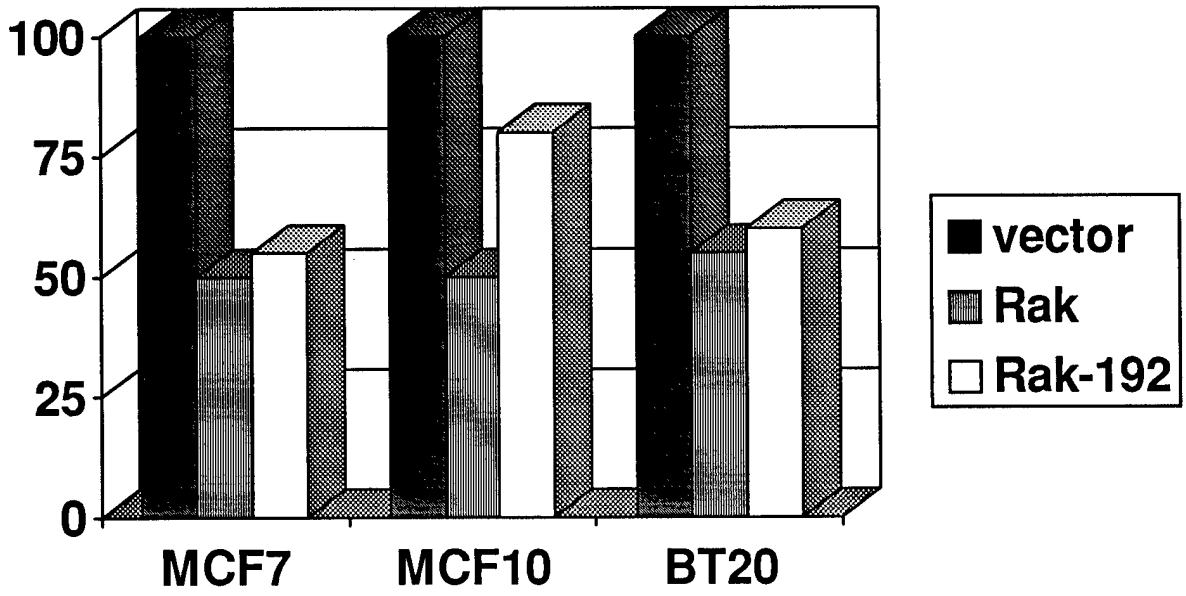

Figure 1. Bar graph showing the results of Rak transfection in three separate breast cancer cell lines. Each bar represents the results from three separate transfections. The procedure used to transfect these cells is explained in the text.

It is interesting to note that the BT-20 and MCF-10 cell lines express Rak, whereas the MCF7 cell line does not. We have confirmed this result both at the protein and RNA level a number of times. We conclude that the growth inhibiting activity of Rak is not limited to Rak-expressing cell lines, but can be generalized to all of the cell lines that we tested. We also found that a kinase inactive mutant of Rak was capable of inhibiting the growth of two of these cell lines.

FUTURE PLANS:

Because this project has undergone a change of Principal Investigator, I would like to submit my plans for the future of this Technical Objective for your review. I feel that the ability of Rak to rapidly inhibit the growth of breast tumor cells warrants further investigation. My lab has developed a large amount of experience with inducible promoters in breast cancer cells, and are presently submitting a manuscript for publication regarding this work. We are able to introduce a potent growth inhibitory gene, a carboxy terminal variant of the Focal Adhesion Kinase, into breast cancer cells and induce its expression. We then monitor expression by immunofluorescence, and analyze the resulting phenotype by TUNEL assay for apoptotic cells and FACS analysis for any block in the cell cycle.

We have already sub-cloned the full-length Rak cDNA into the pMEP4 plasmid and have introduced this plasmid into BT474 breast cancer cells. This cell line was omitted from our earlier analysis for logistical reasons, but we have since developed a series of techniques with this cell line. We also plan to introduce this plasmid into BT-20, MDA231, and MCF7 cells, depending on our initial experiments with the BT474 cells. The pMEP4 plasmid contains a metallothionein promoter, in

which the expression of an inserted gene can be induced by simply adding zinc to the tissue culture medium. This results in minimal toxic side effects, and we have optimized both the selection for cells containing the expression plasmid and the induction conditions.

As implied above, we will assess the induction of Rak gene expression by Western blot analysis with the M2 Flag epitope tag antibody (the Rak cDNA is cloned in frame with the FLAG epitope tag sequence). We will use immunofluorescence with the M2 anti-FLAG antibody to determine the number of induced cells expressing high levels of Rak and to analyze their morphology. Because of our transient transfection experiments, we anticipate that cells will lose adherence following Rak expression and float from the dish. In this case, we will determine whether they have undergone apoptosis by assessing their ability to exclude Trypan Blue, by staining their DNA with Hoechst stain and examining whether they have fragmented nuclei, and by TUNEL assay. If the cells do not appear apoptotic, we will analyze them by FACS analysis to determine whether they have arrested at a particular point in the cell cycle. This is important, because Dr. Liu's lab has already found that expression of Rak in mesenchymal cells and in COS-7 kidney cells causes arrest at the G2/M transition. Thus, G2 arrest is a plausible result for this experiment.

If Rak induces apoptosis or arrest at the G2/M transition, we will return to Technical Objective 1a, which calls for a systematic analysis of the structural features causing this growth arrest. First, we will determine whether growth arrest requires kinase activity by performing the same experiments with a kinase inactive variant of Rak. If this still is capable of inducing G2 arrest, we will try deletion of each of the SH2 and SH3 domains (the structure of Rak is shown in a cartoon diagram in Figure 2). While a number of other experiments are easily imagined along this line, we will await the next results before a more extensive survey of the possible sites of Rak that can be mutated will be reviewed. Our ultimate goal is to determine the nature of the Rak-mediated growth arrest, and assess whether this can be used as a therapeutic target for breast cancers.

Figure 2. The Rak domain structure

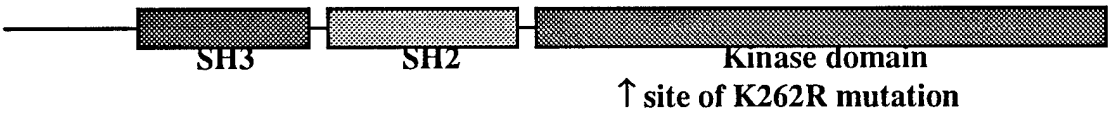

Figure 2. Structural map of the Rak coding sequence, showing the amino-terminal SH2 and SH3 domains. The site for the K262R mutation within the ATP binding pocket of Rak is also shown. This mutation results in a kinase-inactive mutant of Rak.

Our group has attempted to use a yeast system to examine the effect of Rak on the ability of human CDC2 to rescue *cdc28* temperature sensitive yeast phenotype. Our original work suggested that Rak bound to the cell cycle kinase CDC2 through the SH3 domain and that this binding led to the inhibition of CDC2 activity *in vitro*. To test the functional cellular consequences of this binding, we used a yeast strain that carries a temperature sensitive *cdc28* (*Saccharomyces cerevisciae* equivalent of CDC2) mutation and established that constitutive expression of a transfected human CDC2 can rescue the growth arrest seen in the non permissive temperature. We attempted to block the activity of this CDC2 protein with a *rak* expressing plasmid, expecting that if Rak will inhibit human CDC2 it should abrogate the rescue. Our experiments showed that Rak did not affect the ability of CDC2 to

rescue the *cdc28* temperature sensitive phenotype, but we were unable to demonstrate that Rak was expressed in these cells. As a growth inhibitory gene, it is plausible that there may be a counter-selective pressure against the expression of this gene. Indeed, our investigations continue to show that Rak inhibits cells in G2/M and that Rak has both a nuclear and a cytoplasmic localization. Because of the difficulties in these experiments, my lab will not continue to pursue these experiments, but will instead shift our focus to the expression of Rak in breast cancer cells.

B. Technical Objective 2. Determine whether Rak-based sequences can be used to inhibit the Cyclin-dependent kinase CDC2

In Dr. Liu's original proposal, he described work in his laboratory that Rak bound to the CDC2 serine-threonine kinase and inhibited its activity in an *in vitro* kinase assay. Preliminary experiments indicated that an eleven amino acid peptide within the Rak SH3 domain, with the sequence KRRDGSSQQLQ, could by itself inhibit the activity of CDC2. However, this inhibition was weak, with an IC50 in the 100-500 μM range. We attempted to optimize this inhibition by synthesizing shorter peptides containing smaller sequences, such as KRRD or QQLQ, from the original peptide. However, these peptides had less activity that the original 11 amino acid peptide. Specifically, CDC2 was immunoprecipitated from the BT474 breast cancer cell line and was incubated with the appropriate peptide for 30 minutes on ice. An *in vitro* kinase assay was then performed using Histone HI as a substrate. Even with a concentration of peptide ranging up to 1-10 mM, we did not detect a significant decrease in the kinase activity of CDC2. As stated above, the original peptide was only capable of inhibiting CDC2 activity at a similar high concentration. In addition, we obtained our peptides from Research Genetics, and have found a large degree of variance from batch to batch, in which some lots of peptide have minimal inhibitory activity.

Because this work is continuing to be pursued by Dr. Liu's group at the NCI, I do not plan to pursue this Technical Objective. I would prefer to analyze which of the sequences of Rak are required for growth inhibition in breast cancer cells, using the deletion analysis described above. This might reveal new sequences of Rak that are capable of inhibiting growth. If this is the case, I will pursue a similar peptide-based approach to find a small molecule which could be useful as a breast cancer therapeutic.

C. Technical Objective 3. We will determine if Rak is a sensescence gene located on chromosome 6q21.

The experiments described in Objective 3 were not performed. The cell lines described were not obtained or analyzed. At present, we do not plan to pursue this Technical Objective because we lack the reagents and experience to perform these experiments efficiently.

D. Technical Objective 4. Analysis of Cyclin H in human breast cancers

No progress was made on this Objective. My lab is not sufficiently equipped to adequately perform these experiments.

E. Technical Objective 5. Preparation of a transgenic mouse containing the CDK7 and Cyclin H genes under the control of the Whey acid protein (WAP) promoter.

No progress was made on this objective. Instead attempts have been make to create a Rak deficient transgenic mouse through gene disruption. To this end, the putative mouse homologue of Rak, *iyk*,

has been cloned and the 5' most exon identified. Using flanking intronic segments, a plasmid that would disrupt the putative Rak homologue in the first exon has been constructed that contains the promoter and the translational start site. This plasmid has been prepared for introduction into ES cells in collaboration with Dr. Beverly Koller (UNC Transgenic Facility). This work is being pursued in Dr. Liu's lab at the NCI, and will no longer be continued by my lab.

F. Future Plans

Because this grant has changed Principal Investigators in the middle of the funding period, I feel that some adjustments are required for the efficient progress of these experiments. Specifically, I have listed a new plan for the experiments in Technical Objective 1, and propose a revised Technical Objective 2 here.

We do not have a firm understanding of the importance of Rak expression in breast cancer. Our initial characterization of Rak indicated that Rak is expressed in 1/3 of breast cancers at the RNA level. This was a small study, with only 9 samples. Since then, we have used a polyclonal antibody targeted to a GST fusion protein containing the Rak carboxy terminus to study Rak expression in a larger number of samples. At the protein level, we have found that Rak was expressed in 37 out of 100 breast tumors. However, there was a prohibitive amount of background on these blots, making any sort of densitometric quantification difficult.

For this reason, I propose to prepare a monoclonal antibody to Rak. The amino terminus of Rak contains a 57 amino acid unique region which contains only a slight degree of homology to other known proteins. We have prepared a GST fusion protein containing this region for use an immunogen. This protein has been injected into mice, and spleen cells from these mice have been fused to create hybridomas at the North Carolina State Monoclonal Antibody Facility. We have screened 768 hybridoma cultures, and have found 1 isolate which recognizes Rak by Western blot and 1 isolate which recognizes Rak by immunofluorescence on BT474 breast cancer cells. Because Rak is expressed in only a limited number of cell lines, we can readily estimate the specificity of this staining by comparing staining for Rak in BT474 breast cancer cells with staining in a mesenchymally-derived cell line such as the RD rhabdomyosarcoma cell line.

If our preliminary results with these antibodies continue to be positive, we will soon have reagents to study a number of characteristics of Rak. I propose the following objectives:

1. Study the expression of Rak in a large (>100) number of primary breast tumors. We collaborate with Dr. Gary Clark of the University of Texas at San Antonio, and have access to large numbers of breast cancer samples, 200 of which have clinical follow-up. We will analyze Rak expression in cohorts of these samples and correlate the levels of expression with other phenotypical and biological markers of breast cancer which have been measured in these samples. Thus, we can ask, for example, whether Rak expression correlates with clinical stage, outcome, hormonal receptor status, histological grade, ploidy, and S-phase fraction. These will be the first population-based studies of Rak expression and will be performed with appropriated biostatistical support from UNC Lineberger Comprehensive Cancer Center.

2 We have previously found that Rak localizes to the nucleus in a subset of breast tumors and cell lines. I propose to stain tumors which are positive for Rak expression by Western blot by immunohistochemistry and determine whether Rak is localized to the nucleus or the cytoplasm in

these tumors. These experiments will be done on fresh, OCT-embedded breast cancer specimens obtained through the UNC Lineberger Comprehensive Cancer Center Tumor Bank, which I direct. Through these analyses, we can correlate both the levels of Rak expression and the nuclear vs. cytoplasmic localization of Rak with clinical stage, histological grade, and ploidy and cell cycle status. Pending these results, we will then develop methods of immunohistochemical characterization of Rak on formalin-fixed, paraffin-embedded tissues in order to study large samples for more extensive clinical correlations, as described above.

III. REVISED TIME TABLE FOR THE COMPLETION OF WORK

We would propose the following revised time frame for these experiments (given that we are presently at month 18 of the funding period):

Technical Objective 1

a. Isolate and characterize breast cancer cell lines containing a metallothionein promoter: months 18-28.
b. Mutate the kinase domain, and if relevant, the amino-terminal protein interaction domains of Rak and determine their biological activity: months 28-48.

Technical Objective 2

a. Prepare monoclonal antibodies for analysis of Rak in breast tumors: months 18-24
b. Analyze Rak expression in breast tumors by Western blot: months 24-30
c. Analyze Rak expression and localization in breast tumors by immunohistochemistry: months 24-48.

Technical Objective 3

a. Screen for deletions of the Rak gene in DNA of breast tumors (dependent on the results from Technical Objective 2b): months 30-48

IV. CONCLUSIONS

These experiments are still in the preliminary phases, but we have shown that Rak appears to be an important tyrosine kinase in human breast cancer. We have found that Rak has a growth inhibitory effect on breast cancer cells, and have proposed a series of experiments to further refine these findings. Our goal is to develop therapeutic and diagnostic strategies for breast cancer. To address the latter objective, we are preparing monoclonal antibodies to the amino terminus of Rak, which we will use to assess the expression and localization of Rak in clinical breast tumor specimens.

V. REFERENCES

During this initial year there were no publications generated from this project. Portions of this work were presented at the Department of Defense "Era of Hope" annual meeting in Washington, D.C. on November 2, 1997 as a platform presentation in the Cell Cycle Mini-symposium by Dr. Rolf Craven.

VI. APPENDICES

None

VII. LIST OF PERSONNEL

Olga Aprelikova
Elizabeth M. Bowers
William G. Cance
Subhashini Chandrasekharan
Yan Lin
Chunru Lin